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Review

Translational regulation of ornithine decarboxylase and other enzymes of the polyamine pathway

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Abstract

It has long been known that polyamines play an essential role in the proliferation of mammalian cells, and the polyamine biosynthetic pathway may provide an important target for the development of agents that inhibit carcinogenesis and tumor growth. The rate-limiting enzymes of the polyamine pathway, ornithine decarboxylase (ODC) and *S*-adenosylmethionine decarboxylase (AdoMetDC), are highly regulated in the cell, and much of this regulation occurs at the level of translation. Although the 5' leader sequences of ODC and AdoMetDC are both highly structured and contain small internal open reading frames (ORFs), the regulation of their translation appears to be quite different. The translational regulation of ODC is more dependent on secondary structure, and therefore responds to the intracellular availability of active eIF-4E, the cap-binding subunit of the eIF-4F complex, which mediates translation initiation. Cell-specific translation of AdoMetDC appears to be regulated exclusively through the internal ORF, which causes ribosome stalling that is independent of eIF-4E levels and decreases the efficiency with which the downstream ORF encoding AdoMetDC protein is translated.

The translation of both ODC and AdoMetDC is negatively regulated by intracellular changes in the polyamines spermidine and spermine. Thus, when polyamine levels are low, the synthesis of both ODC and AdoMetDC is increased, and an increase in polyamine content causes a corresponding decrease in protein synthesis. However, an increase in active eIF-4E may allow for the synthesis of ODC even in the presence of polyamine levels that repress ODC translation in cells with lower levels of the initiation factor. In contrast, the amino acid sequence that is encoded by the upstream ORF is critical for polyamine regulation of AdoMetDC synthesis, and polyamines may affect synthesis by interaction with the putative peptide, MAGDIS. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Ornithine decarboxylase; *S*-Adenosylmethionine decarboxylase; Translational regulation; Polyamines; eIF-4E

Abbreviations: ODC, ornithine decarboxylase, AdoMetDC, *S*-adenosylmethionine decarboxylase, SSAT, spermidine/spermine *N*¹-acetyltransferase, PLP, pyridoxal 5'-phosphate, PAO, polyamine oxidase, UTR, untranslated region, ORF, open reading frame, TPA, 12-*O*-tetradecanoylphorbol-13-acetate, DFMO, α -difluoromethylornithine, BE-3-

4-3, *N*¹,*N*¹²-bis(ethyl)spermine, BDAP, *n*-butyl-1,3-diaminopropane, AbeAdo, 5'-{[(*Z*)-4-amino-2-butenyl]methylamino}-5'-deoxyadenosine.

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1. Introduction

The polyamines putrescine, spermidine and spermine have been shown to be essential for cell growth and differentiation, and depletion of polyamines inhibits growth of neoplastic cells both in vitro and in animal models. The polyamine-metabolizing enzymes are activated by a variety of growth factors, carcinogens, viruses and oncogenes, and the cellular activities of these enzymes are very tightly controlled. The polyamine biosynthetic pathway is shown in Fig. 1. The first step in polyamine biosynthesis is the formation of putrescine from ornithine and is catalyzed by ODC. ODC is active as a dimer, which contains two active sites formed at the interface between monomers [1,2]. ODC has been found to be dependent on pyridoxal 5'-phosphate (PLP) for activity from all sources so far isolated. The PLP binding site, as well as other key active site residues for ODC, have been identified [1–3].

The higher polyamines spermidine and spermine are formed by the successive transfer of two aminopropyl groups. The aminopropyl donor for these reactions is decarboxylated *S*-adenosylmethionine, which is formed by the action of AdoMetDC on *S*-adenosylmethionine. This is also an important step because once *S*-adenosylmethionine is decarboxylated it is committed to a role in polyamine biosynthesis and cannot be used for the various methylation reactions for which it is normally a substrate. AdoMetDC is synthesized as a proenzyme which then undergoes an internal processing reaction forming α and β subunits and a pyruvate prosthetic group, which is located at the amino terminus of the α subunit [4]. The processing and activity of AdoMetDC are increased by putrescine, providing a means by which the increased availability of putrescine raises the formation of decarboxylated AdoMet which is needed for the next enzyme in the biosynthetic pathway, spermidine synthase, to convert putrescine into spermidine [5,6]. The aminopropyl transfer needed to form spermine from spermidine is carried out by spermine synthase.

Because the biosynthetic pathway is essentially irreversible, a back reaction exists in which sper-

midine and spermine are first acetylated by spermidine/spermine *N*¹-acetyltransferase (SSAT) and then oxidized by polyamine oxidase (PAO). SSAT is the rate-limiting enzyme of this catabolic pathway, and exposure to high levels of polyamines produces an increase in intracellular SSAT activity [7]. The effect of induction of SSAT is to reduce the content of spermidine and spermine in the cell, and therefore SSAT acts as a homeostatic mechanism in the cell to prevent the accumulation of polyamines to cytotoxic levels.

2. Regulation occurs on many levels

The levels of ODC, AdoMetDC and SSAT show striking changes in response to factors affecting cell growth and to changes in polyamine levels, and extensive alterations in enzyme activities can occur very quickly. These alterations are usually the result of changes in the absolute amount of enzyme protein [5,8] and regulation can occur at the levels of transcription, translation and protein degradation. For example, the promoters of the genes encoding ODC and AdoMetDC contain multiple regulatory elements, and there are numerous examples of transcriptional regulation of both genes by growth factors, hormones, and tumor promoters.

Increased levels of mammalian AdoMetDC mRNA, which presumably are due to changes in transcription, have been seen in response to growth factors and to a decline in spermidine produced by a variety of inhibitors [5,9–17]. Insulin also increases AdoMetDC mRNA synthesis and an insulin-responsive element was found in the rat AdoMetDC promoter [18].

The ODC gene is considered an immediate early gene, and contains response elements for several *trans*-acting factors, including a cAMP response element, a possible insulin response element and several Sp1 binding sites. Although the ODC promoter is known to respond to the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a TPA-responsive element has not been positively identified, and it has been suggested that regulation of ODC by TPA is cell-type specific [19–22]. Transcriptional regulation

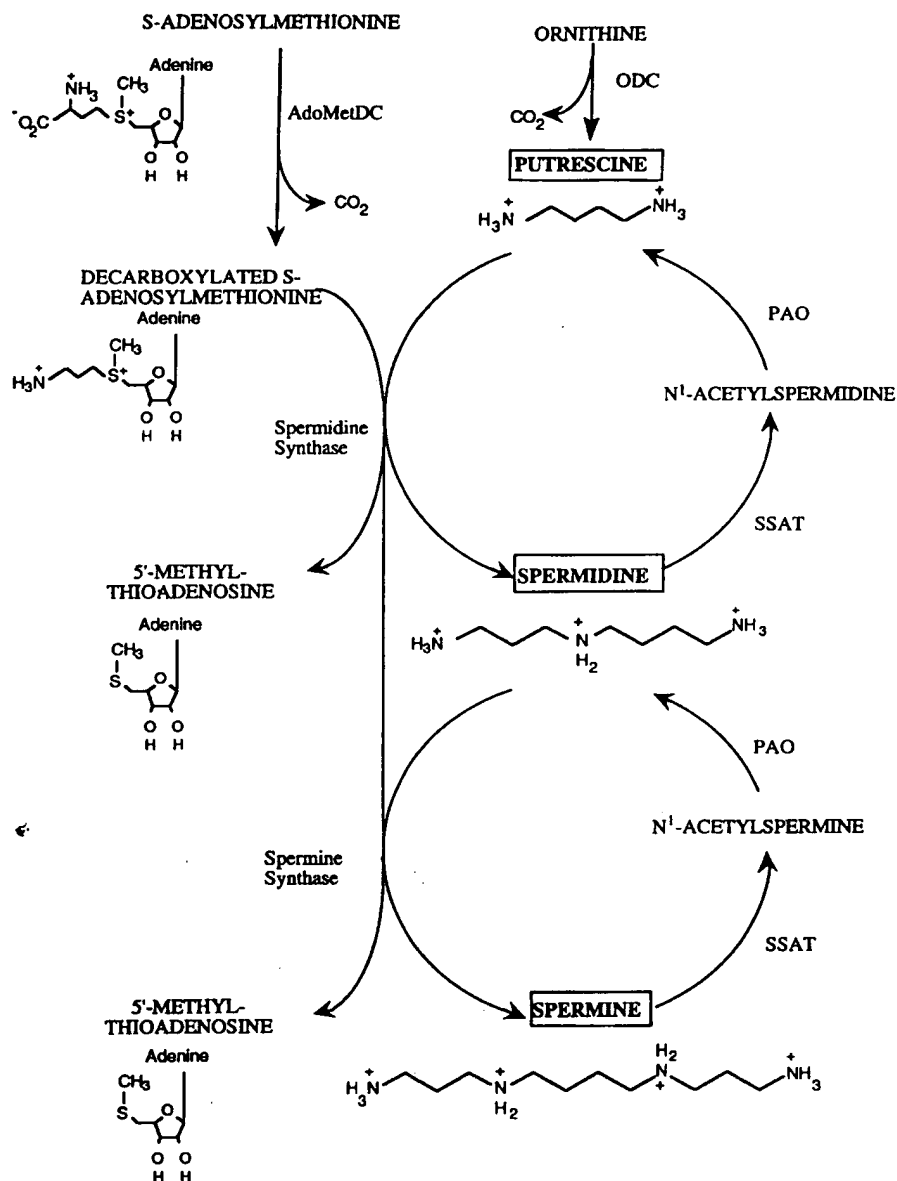


Fig. 1. The polyamine metabolic and catabolic pathways. The abbreviations of the enzymes are as follows: ODC, ornithine decarboxylase; AdoMetDC, S-adenosylmethionine decarboxylase; SSAT, spermidine/spermine N¹-acetyltransferase; PAO, polyamine oxidase.

of the ODC gene by many of these elements has been reviewed recently [23]. In addition, induction of the ODC gene may play a role in the signaling pathways of several oncogenes. Transformation by activated *ras*, *v-src* and *myc* appears to be tightly coupled to ODC gene expression and polyamine accumulation [24–28], and ODC is a transcriptional target of c-Myc [29–33]. Massive overexpression of ODC in 3T3 cells and 10T1/2 cells leads to transformation [34–36], suggesting that, under some conditions, the ODC gene may act as an oncogene. ODC-overproducing cells produce large, highly vascularized tumors in nude mice, and recent results have suggested that ODC overproduction may lead to the increased secretion of angiogenesis factors [37, 38].

At the post-translational level, the intracellular half-lives of ODC, AdoMetDC and SSAT are less than 1 h. At present, the mechanism for the degradation of AdoMetDC and for the alterations in degradation seen in response to polyamines, inhibitors and other stimuli are unknown. On the other hand, the degradation of SSAT and especially ODC have been well studied in recent years. The degradation of ODC requires a domain located in its carboxyl end and has been shown to be controlled by a protein termed antizyme, which binds to ODC and renders it susceptible to degradation by the 26S proteasome [39–42]. An example of the importance of ODC degradation as a means of regulation during neoplastic transformation is demonstrated in studies on hepatocellular carcinoma. It has been shown that ODC from rat liver preneoplastic nodules and hepatoma is more stable than that from normal liver tissue [43]. More recently, point mutations in the ODC gene which render the protein more stable have been detected in human hepatocellular carcinoma [44]. Recent work from our laboratory has demonstrated that the SSAT protein is a good substrate for degradation via the proteasomal/ubiquitin pathway, possibly through interaction of the proteasome with the carboxyl end of SSAT [45, 46]. Although the activity of both enzymes leads to an increase in putrescine in the cell, ODC and SSAT have opposing roles in polyamine synthesis, with ODC

tending to increase and SSAT to decrease polyamine levels [5, 8]. Both enzymes are regulated at the level of protein stability by the polyamines, but in opposite directions. ODC content is reduced by polyamines via the antizyme-mediated enhancement of degradation, whereas SSAT is increased by polyamines via the prevention of degradation.

Although the regulation of the enzymes of the polyamine pathway is obviously quite complex and interdependent, the remainder of this review will concentrate on the translational regulation of the polyamine-metabolizing enzymes, ODC and AdoMetDC. The emphasis will be on ODC, and its translational regulation during neoplastic transformation.

3. Translational regulation of ODC synthesis

3.1. Secondary structure

In the study of translational regulation of ODC, much of the work has focused on the long 5' untranslated region (5'UTR) of its mRNA. Although most genes encode mRNAs with short unstructured 5'UTRs, oncogenes and genes involved in cellular proliferation frequently encode mRNAs with long 5'UTRs that are predicted to form extensive secondary structure [47, 48]. The predicted secondary structure of the 5'UTR of mouse ODC is shown in Fig. 2. The ODC cDNAs cloned from a variety of species all have long, highly conserved 5'UTRs of 275–313 nt. In all cases, this 5'UTR is predicted to form an extensive secondary structure (ΔG of approximately -115 kcal/mol). It also contains a small internal ORF located about 150 nt 5' to the AUG initiation codon. Both of these features have been shown to reduce translational efficiency in other mRNAs [50, 51]. In rabbit reticulocyte lysates, translation of both mouse and rat ODC containing the full length 5'UTR was reduced by 95% compared to mRNA containing very short 5' leader sequences [52–55]. Furthermore, a conserved GC-rich region in the 5'-most 125–130 nt of the ODC 5'UTR repressed translation to the same extent as the entire

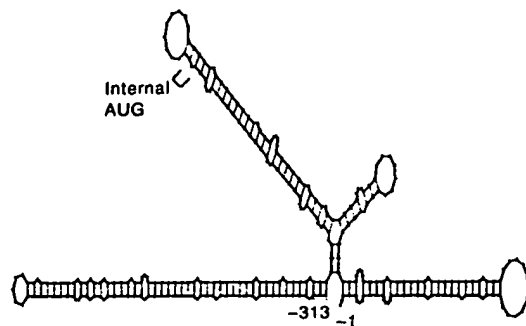


Fig. 2. The 5'UTR of mouse ODC taken from Ref. [49]. The sequence of the 5'UTR begins at nt -313 and ends at nt -1. The AUG of the internal ORF, which begins at nt -157 in the mouse sequence, is indicated. The predicted ΔG of the structure is -115 kcal/mol.

5'UTR [52, 55]. The plasmids used in these experiments also contained the full length ODC 3'UTR (326 nt in the mouse). Similarly, in cultured cells, the synthesis of reporter genes was inhibited by up to 99% when the full length ODC 5'UTR from rat, mouse or hamster was inserted immediately before the initiation codon [52, 56, 57]. In agreement with the *in vitro* experiments, the GC-rich 5' end of the leader sequence was almost as effective as the full length 5'UTR in suppressing protein synthesis [52, 56, 58]. This region is predicted to form a stable stem-loop structure with a calculated ΔG of -66 kcal/mol in the mouse [59].

Another factor which may play a role in the translational regulation of ODC is a possible interaction of the 5'UTR and the 3'UTR of the ODC mRNA. As mentioned above, the 3'UTR of ODC is quite long, and it has been shown that the absence of a 3'UTR decreased the expression of an ODC construct containing the full-length 5'UTR by 64% *in vitro* [52]. *In vivo*, Scheffler and colleagues have shown that the 3'UTR was able to partially release the inhibition of luciferase expression caused by the 5'UTR in transiently transfected CHO cells and COS-1 cells, as well as in stably transfected CHO cells. A 6- to 30-fold increase in translation was observed relative to a comparable construct containing only

the 5'UTR [56, 60]. Interestingly, this effect could not be duplicated when translating the same luciferase plasmids in a rabbit reticulocyte lysate system, which led to the suggestion that cellular factors are responsible for an interaction between the ODC 5' and 3'UTRs, and these factors were either not present in the reticulocyte lysates used, or were present in an inactive state [60]. In support of this idea, the addition of cytoplasmic extracts from serum-stimulated cells significantly increased the *in vitro* translation of the luciferase mRNA containing both the ODC 5' and 3'UTR's, while the effect on the translation of mRNA containing only the 5'UTR was minimal [60].

Any protein factors that may interact with either the 5' or 3'UTR of ODC remain unidentified, although it has been reported that a 58 kDa protein specifically binds to a conserved heptanucleotide region (CCAU/ACUC) of the ODC 5'UTR, located approximately 60 nt from the initiation codon [61]. In addition, it has been shown recently that alternatively spliced ODC 5'UTR's exist in tumor-derived pancreatic acinar AR4-2J cells [62]. These variants result from the lengthening of the 5' end of exons 2 and 3 by 17 and 13 bases, respectively, and increase the rate of ODC translation efficiency up to 8-fold. These results also support the idea of *trans*-acting regulatory factors binding to specific sequences within the 5' leader sequence.

3.2. Internal ORF

As mentioned above, the cDNAs of all ODC species identified to date contain a small ORF within the 5'UTR. The sequence around this internal ORF lacks the -3 purine but does contain a G residue in position +4 thought to be necessary for efficient translation [51, 63]. The predicted peptide sequence is not as conserved as that of the AdoMetDC internal ORF (discussed below), ranging from 10 amino acids in the rat, mouse and human, to 38 amino acids in the hamster. The internal AUG is contained within the 3'-most 160 nt of the ODC 5'UTR, which contains less secondary structure than the GC-rich 5' end. Several groups have attempted to assess the con-

tribution of the ODC internal ORF by constructing plasmids containing point mutations within the AUG, which would prevent translation of the internal ORF. Several of these plasmids also contained deletions in the 5'UTR, however, making interpretation of the results difficult. For example, when the sequence from positions –160 to –1 was attached to either the growth hormone or luciferase reporter mRNA, translation was repressed by about 50% and this effect was abolished by the mutation of the internal AUG initiation codon [52,56]. However, the 50% inhibition seen with the AUG intact was far less than the 95% inhibition brought about by the full length 5'UTR.

Two reports have used plasmids containing the full length ODC 5'UTR in which the internal ORF has been destroyed. In studies expressing luciferase in NIH-3T3 cells using a construct in which the ODC 5'UTR is fused to the luciferase coding region, the internal ORF in the ODC 5'UTR was destroyed by an A to C point mutation [58]. Although this plasmid contained the same predicted secondary structure as the wild type 5'UTR plasmid, its level of expression was approximately 5-fold greater than that of the wild type 5'UTR, and was comparable to that of a construct containing only the 3'-most 10 nt of the ODC 5'UTR. In contrast, *in vitro* translation of ODC plasmids containing the full length 5'UTR with or without an A to U point mutation in the internal ORF showed no difference in translation efficiency, which was very poor [55]. This again may point to a difference between *in vivo* and *in vitro* experiments, in which some cellular factor responsible for the release of translation inhibition is limiting in reticulocyte lysates. In any case, the contribution of the internal ORF to ODC translational regulation, as well as any factors that may interact with it, is the subject of continuing study.

3.3. Regulation by translation initiation factors

Because the secondary structure of the ODC 5'UTR is a factor in its translational regulation, it is reasonable that the translation of ODC might be regulated by the activation of trans-

lation initiation factors that melt this secondary structure. For example, insulin induces ODC activity and stimulates the phosphorylation of the RNA-binding initiation factor eIF-4B and the cap-binding protein eIF-4E, which are involved in mRNA binding to the 40S initiation complex, usually the rate-limiting step in translation initiation [64]. Phosphorylation of these factors is increased by mitogenic stimuli and it has been suggested that eIF-4E, the least abundant of the factors which make up the eIF-4F complex, plays a regulatory role in translation [50]. Interestingly, only the 5'-most 115–130 nt of the 5'UTR, which contain most of the secondary structure, were necessary for the stimulatory effect of insulin on ODC translation [64]. Other investigators have shown an increase in eIF-4E phosphorylation and eIF-4A helicase activity in ODC-overexpressing mouse mammary tumor cells [65]. The ODC overproduction in these cells was in response to chronic exposure to the ODC inactivator α -difluoromethylornithine (DFMO) [66].

The importance of eIF-4E in growth control has been demonstrated by Sonenberg and colleagues in NIH-3T3 cells overexpressing eIF-4E (pMV7-4E(P2) cells; 4E-P2 cells), which exhibit characteristics of cellular transformation, including formation of transformed foci on a monolayer, anchorage-independent growth and tumor formation in nude mice [67]. mRNAs containing extensive secondary structure in their 5'UTRs translate more efficiently in cells overexpressing wild type eIF-4E than an inactive eIF-4E [68]. It has also been shown that eIF-4E can cooperate with Myc and E1A in the transformation of primary embryo fibroblasts [69], and reduction in the level of eIF-4E in rat embryo fibroblasts using antisense plasmids caused reversion of transformation by oncogenic *ras* [70,71]. Other recent results have shown that overexpression of eIF-4G can also result in malignant transformation of NIH-3T3 cells [72]. eIF-4G, another component of the eIF-4F complex, acts as a scaffold protein, and interacts with eIF-4E, the RNA helicase eIF-4A and eIF-3 [73,74]. The presence of eIF-4G increases the affinity of eIF-4E for the cap structure [75].

Both DFMO treatment and expression of an ODC dominant negative mutant abolish the transformed phenotype of cells overexpressing eIF-4E, suggesting strongly that ODC is important in the neoplastic response [57, 76]. However, it is likely that other growth factors/oncogenes combine with ODC to lead to transformation. For example, p21^{ras} protein is activated in 4E-P2 cells, as shown by an increased proportion of GTP-bound p21^{ras}, although no increase in the synthesis of p21^{ras} was observed [77]. In addition, overexpression of a negative regulator of cellular p21^{ras}, GTPase activating protein (RasGAP), causes reversion of the transformed phenotype [77]. However, the relationship between Ras activation and ODC induction in these cells remains to be determined.

eIF-4E is thought to be regulated by the binding of 4E-BP1 (also called PHAS-I). 4E-BP1 regulates translation by competing with eIF-4G for an eIF-4E binding site, resulting in the formation of an inactive complex between 4E-BP1 and eIF-4E [78–81]. Phosphorylation of 4E-BP1 occurs in response to growth-promoting stimuli such as insulin, EGF and PDGF, all of which induce ODC. eIF-4E is then released and becomes available to form an active cap-binding complex [74]. Recently, the overexpression of 4E-BP1 was found to cause a partial reversal of the transformed phenotype in 4E-P2 cells [82]. In contrast, however, disruption of the 4E-BP1 gene in mice produced no remarkable phenotype and the ODC level in these mice was not statistically different from controls [83]. The presence of a functional homologue of 4E-BP1, called 4E-BP2 or PHAS-II, may account for these results [79, 84, 85].

The 4E-P2 cells provide an excellent model for studying the regulation of translation of ODC by eIF-4E and the possible interaction of this factor with polyamines in influencing the translation of ODC mRNA. These cells exhibit high endogenous levels of ODC (up to 30 times that in 3T3 cells) without a rise in RNA levels, strongly suggesting translational regulation of ODC by eIF-4E [57]. Studies using polysomal distribution of ODC mRNA have confirmed that translation initiation of intracellular ODC

is increased in 4E-P2 cells [86]. Additional evidence that the intracellular availability of active eIF-4E correlates with ODC translation has been provided in studies using IEC-6 intestinal epithelial cells, where it has been shown that rapamycin, which blocks phosphorylation of 4E-BP1, inhibits the induction of ODC in response to serum [87]. ODC translation is also decreased upon expression of antisense eIF-4E in *ras*-transformed rat embryo fibroblasts [88].

Other recent studies in 4E-P2 cells have used plasmids containing deletion mutants of the ODC 5'UTR with decreasing degrees of predicted secondary structure inserted directly upstream of the luciferase protein coding region, shown in Fig. 3 [58]. ODC-luciferase plasmids with increasing secondary structure in their 5'UTRs were expressed better in 4E-P2 cells than in the parental NIH-3T3 cells in all cases, suggesting that the secondary structure inhibits ODC expression in 3T3 cells and this inhibition is overcome by high eIF-4E levels in 4E-P2 cells. However, the level of increased luciferase expression of a plasmid containing the full length 5'UTR was significantly less than the increase in endogenous ODC in 4E-P2 cells compared to NIH-3T3 cells. In fact, the largest increase in luciferase expression from the ODC 5'UTR-luciferase constructs, which was seen in both 4E-P2 cells and NIH-3T3 cells, was the result of destroying the internal ORF in the ODC 5'UTR by a point mutation (plasmid ODC313-ATG in Fig. 3). This is consistent with the scanning model of translation in which the presence of an internal ORF can cause an inhibition of translational initiation [47, 51, 63], and suggests that the translational machinery stalls at this point in the chimeric plasmids but not in the endogenous ODC mRNA. These results point to another means of reducing translation which may be overcome in 4E-P2 cells. For example, the unwinding of secondary structure caused by high levels of eIF-4E may enhance the interaction of 5'UTR and 3'UTR sequences, thereby overcoming both the secondary structure and internal ORF inhibition of ODC translation.

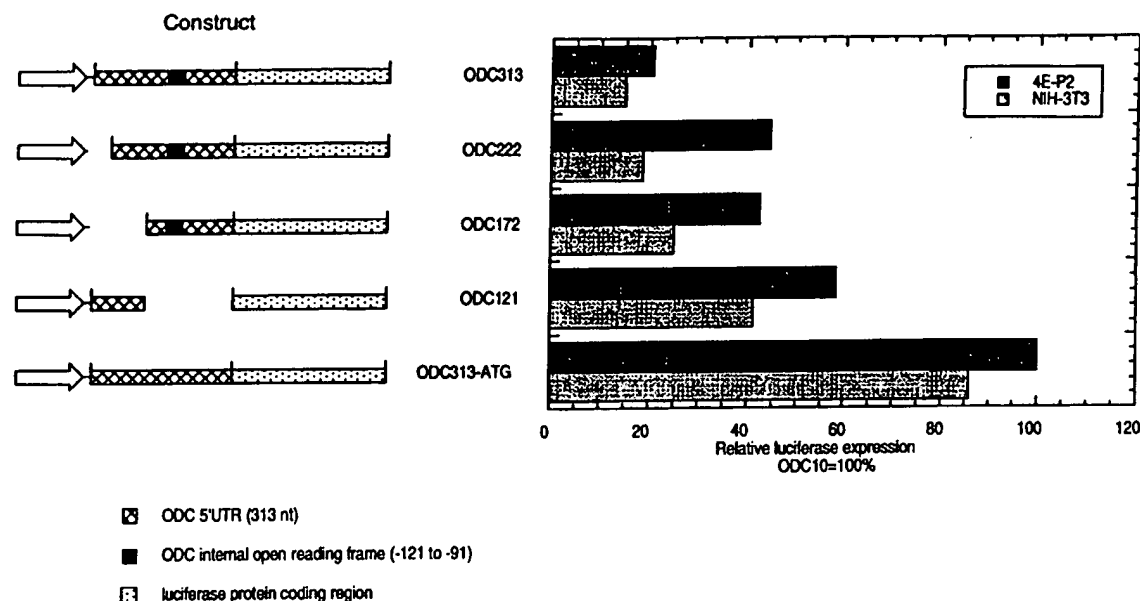


Fig. 3. Expression in 4E-P2 and 3T3 cells of ODC-luciferase constructs with varying degrees of secondary structure in the ODC 5'UTR. The results are taken from Ref. [58]. All results are corrected for transfection efficiency by dividing by the CAT activity obtained from the transfection of pSV-CAT. The ODC10 plasmid, which contains nt -10 to -1 of the ODC 5'UTR, was taken to be 100% in each cell line and the results are expressed as a percentage of this value.

3.4. Regulation by polyamines

The second major factor known to influence the translational efficiency of ODC mRNA is the intracellular content of polyamines. Low levels of polyamines are necessary for general protein synthesis but excess polyamine levels inhibit the translation of most mRNAs. ODC synthesis, however, is much more sensitive to polyamine regulation, and is stimulated at lower concentrations of polyamines than general protein synthesis [53,89,90]. Inhibition of ODC translation also occurs at relatively low levels of polyamines compared to general translation of proteins, and an increase in the content of polyamines has been shown to reduce ODC translation both in reticulocyte lysates [53,54,89,90] and in cells in culture [66,91,92]. Spermidine and spermine are more potent regulators of translation *in vitro* than putrescine, with stimulation of ODC synthesis optimal at about 200 μ M spermidine and inhibition occurring at concentrations above

400 μ M, which is within the physiological range [53,89,90,93,94]. Exposure to polyamine analogues has a similar effect in repressing ODC synthesis without affecting mRNA content [95].

Removing or truncating the 5'UTR from the ODC mRNA abolishes the polyamine effect [53,54,92]. However, the exact location of any polyamine responsive element in the 5'UTR of ODC has yet to be defined. The results of Ito et al. [53] suggest that the response of ODC to spermidine is mediated through a sequence between nt -70 and -170. Mutation of the internal ORF, which is within this sequence, had little effect on the regulation by spermidine [54]. On the other hand, inhibition of the translation of ODC mRNA by bis(ethyl)polyamines in reticulocyte lysates was manifest even with an mRNA that contained only nt -69 to -1 of the 5'UTR [95], and as discussed above, this is the same region in which a protein binding domain has been located [61]. This suggests that the region responsible for the polyamine effect is located within

the 3'-most sequence of the 5'UTR. It is interesting that several reports show that ODC mRNA containing only the 3'-most 69–71 bases (nt –71 to –1) are actually translated slightly better than mRNA containing shorter sequences [52,55]. It has also been shown with other cDNAs that translation increases proportionally as the distance between the cap and the ORF is increased from 17 to 80 nt, but these studies used random sequences with very little secondary structure [63].

Several experiments have failed to detect any effects of polyamines on translation of ODC mRNA in vitro [55] or reporter constructs containing the ODC 5'UTR in vivo [56]. In addition, two studies have reported in vivo regulation of constructs containing the ODC protein coding region, but no 5'UTR sequence, although the 3'UTR was intact [96,97]. It appears, however, that the conditions used in at least some of these experiments may stimulate the degradation of the ODC, making results difficult to interpret [92].

It is likely that the regulation of ODC is cell-specific, and that intracellular levels of other factors combine with changes in polyamines to influence translation. For example, in examining the difference in response of ODC to polyamines in 4E-P2 and NIH-3T3 cells, it was found that the polyamine analogue N^1,N^{12} -bis(ethyl)spermine (BE-3-4-3) down-regulates the ODC activity in both 4E-P2 cells and 3T3 cells in a time- and dose-dependent manner. The ODC in 4E-P2 cells is however significantly more resistant to modulation by BE-3-4-3 than the ODC in 3T3 cells, suggesting that the regulation of ODC levels by polyamines is decreased in 4E-P2 cells [58]. Intracellular levels of all the polyamines average about 1.5–2 times higher in 4E-P2 cells compared to 3T3 cells, which would be predicted to result in decreased ODC expression, yet ODC levels remain elevated in 4E-P2 cells [57]. It is also noteworthy that the translation of ODC mRNA is increased in mitogen-activated lymphocytes, as shown by a shift of ODC mRNA from untranslated particles onto polysomes [98]. Since mitogen activation causes polyamine elevation, this would also be expected to lower rather than raise the translational efficiency of the ODC mRNA [98]. However, there is an increased ac-

tivity of eIF-4F due to phosphorylation of eIF-4E within 30 min of mitogen treatment [99].

In summary, these results suggest that any cell specific regulation of ODC may be the result of different polyamine and phosphorylated eIF-4E levels among different cell types. In cell types or under conditions that limit the amount of active eIF-4E, ODC translation may respond more dramatically to changes in polyamine content. Thus, when polyamine levels are low, an interaction between the 5' and 3'UTRs, which perhaps involves other cellular factors, relieves the secondary structure of the 5' leader (and perhaps masks the internal ORF), allowing for more efficient ribosomal scanning. High levels of polyamines, however, interfere with this interaction, either directly or through the synthesis of other regulatory proteins. An increase in active eIF-4E overcomes the polyamine effect, allowing for the synthesis of ODC at polyamine concentrations that would suppress ODC translation in cells with lower levels of the initiation factor. This is consistent with the observations that both ODC and eIF-4E are elevated in many transformed cell lines, allowing for efficient translation of ODC even as polyamine levels accumulate.

4. Translational regulation of AdoMetDC synthesis

In virtually all of the circumstances reported in which enhanced levels of AdoMetDC mRNA have been observed, these increases in mRNA are insufficient to account for the increases in AdoMetDC protein content [4,9,11,12,14,15,100]. The translational regulation of AdoMetDC has been reviewed within the context of several more general reviews [4,101,102]. The emphasis in this chapter, therefore, will be a comparison between the translational regulation of ODC and that of AdoMetDC.

All mammalian AdoMetDC mRNAs contain a long 5'UTR of about 330 nucleotides, the sequence of which is very highly conserved [9,103–106]. This has focused more detailed biochemical studies on the translational regulation

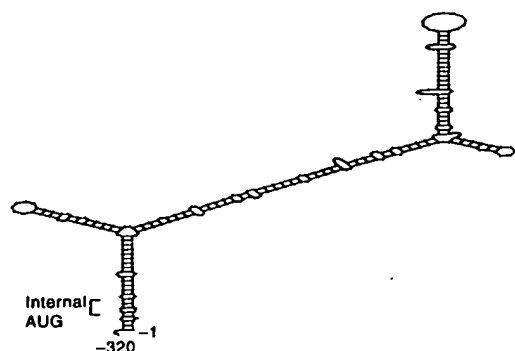


Fig. 4. The 5'UTR of human AdoMetDC taken from Ref. [107]. The sequence of the 5'UTR begins at nt -320 and ends at nt -1. The AUG of the internal ORF, which begins at nt -309 in the human sequence, is indicated. The predicted ΔG of the structure is -69 kcal/mol.

towards the possible effect of this 5'UTR sequence. The 5'UTR of human AdoMetDC is shown in Fig. 4. The ΔG of the predicted secondary structure is -69 kcal/mol. Although this is less stable than the secondary structure of the ODC 5' leader, stem-loop structures with ΔG s of -50 to -60 kcal/mol may be capable of inhibiting translation initiation at downstream AUG codons [51]. As discussed above, the GC-rich 5' end of the ODC leader sequence, with a calculated ΔG of -66 kcal/mol, was almost as effective as the full length 5'UTR in suppressing protein synthesis. Like the 5'UTR of ODC, the AdoMetDC leader contains a small internal ORF. Unlike ODC, however, the internal ORF of AdoMetDC is located very close to the cap (11–14 nt), is identical among all reported mammalian cDNAs and is in perfect context for translation [63]. The sequence around the ORF is CACAGTATGG with the initiation codon italicized [104, 108].

Direct evidence for translational regulation of AdoMetDC synthesis was first obtained by pulse labeling studies [15, 109] and by examining the polysomal distribution profiles of the endogenous AdoMetDC mRNA [110]. Translational regulation by the 5' leader sequence of AdoMetDC has been examined in experiments in which mammalian cells were transfected with plasmids containing full length AdoMetDC mRNA with or without deletions in the 5'UTR, or reporter

genes fused to the 5'UTR [100, 104, 105, 107, 108]. The results indicated that AdoMetDC is much more efficiently translated in non-lymphoid cell lines, in which the mRNA associates with polysomes, than in cells of lymphoid origin, where most of both the endogenous AdoMetDC mRNA and the mRNA of reporter genes fused to the AdoMetDC 5'UTR is found associated with monosomes [104, 105, 108]. In addition, the secondary structure in the 5'UTR was not a key factor in translational regulation in any cell line studied [100, 104, 105, 107, 108]. Indeed, removing only the 5'-most 27 nt of the 5'UTR resulted in a similar increase in expression as removing virtually the entire 5'UTR [107].

Other experiments compared translational regulation of AdoMetDC as well as luciferase constructs containing the AdoMetDC 5'UTR in NIH-3T3 cells and 4E-P2 cells. Unlike ODC activity, which was induced 30-fold in 4E-P2 cells, endogenous AdoMetDC activity was not different in 4E-P2 cells compared to NIH-3T3 cells. Similarly, there was no difference in luciferase expression from the constructs containing the AdoMetDC 5'UTR between the two cell types, and this expression was very poor [57]. These results provide additional evidence that reading of the AdoMetDC mRNA is not dependent on secondary structure and is not regulated by eIF-4E.

Although there is an intriguing report of a protein binding to the 5'UTR at a region located in the 52 nucleotides at its 3'-most end [106], the sequence responsible for the translational regulation of AdoMetDC appears to be located very close to the 5' end of the 5'UTR and has been identified as the small internal ORF, which would code for a 6 amino acid peptide. Mutation of this ORF to abolish the initiation codon greatly increased translation of the downstream AdoMetDC ORF, suggesting that translation of the upstream ORF was a critical regulatory feature [104, 105, 107, 108]. In addition, translation of a plasmid deleting nt -288 to -12 of the human 5'UTR, but leaving the internal ORF intact (from nt -309 to -289), expressed almost as poorly as the wild type construct, even though the predicted secondary structure had been

reduced from -69 kcal/mol to -5.8 kcal/mol [107]. The upstream ORF was also more effective in suppressing translation in T lymphocytes compared to adenocarcinoma cells and fibroblasts, thus accounting for the cell specific effects in AdoMetDC synthesis [104,105]. This effect is related to the position of the ORF close to the message cap site, an effect which is likely to be due to a difference in efficiency of recognition and initiation at the upstream ORF among different cell types.

Several experiments have shown directly that the translation of mammalian AdoMetDC mRNA is very effectively repressed by polyamines. In vitro studies on this repression were carried out using reticulocyte lysates and human AdoMetDC mRNA [89,111,112]. Several in vivo experiments made use of inhibitors of either ODC (DFMO) or AdoMetDC itself (5'-{[(Z)-4-amino-2-butenyl]methylamino}-5'-deoxyadenosine, AbeAdo) to manipulate intracellular polyamine levels and examine the changes in AdoMetDC mRNA and protein in L1210 leukemia cells [14,109] or Swiss 3T3 fibroblasts [110]. Cells treated with such inhibitors responded by increasing their levels of AdoMetDC protein, as measured by increased incorporation of [35 S] methionine in AbeAdo-treated cells [14,109], or increased AdoMetDC activity in DFMO-treated cells [110]. However, in no case was the increase in AdoMetDC mRNA sufficient to account for the increase in protein, suggesting translational regulation. Indeed, the AdoMetDC mRNA in DFMO-treated cells was found to be associated almost entirely with large polysomes, whereas much of the AdoMetDC mRNA in untreated cells was associated with monosomes, suggesting that polyamine depletion increases the translational initiation of AdoMetDC [110].

Other in vivo experiments utilized constructs containing the coding region for AdoMetDC and the entire 3'UTR, with mutations and/or deletions in the 5'UTR [100,107]. By varying the intracellular spermine concentration using the spermine synthase inhibitor *n*-butyl-1,3-diaminopropane (BDAP), it was shown that a spermine response element resides in the first 27 nt of the AdoMetDC 5'UTR, which contains the internal

ORF. In addition, the secondary structure of the AdoMetDC 5'UTR was not a factor in spermine regulation, since deletion of nt -288 to -12 did not interfere with this regulation [107]. More elegant studies have been performed in which the 5'UTR of AdoMetDC has been fused to a reporter gene. These studies confirmed that the 5'UTR of the AdoMetDC mRNA contains a polyamine responsive element which reduces translation in the presence of spermidine or spermine, and showed that this element resides entirely within the internal ORF sequence [107,113]. On the other hand, in experiments utilizing plasmids coding for AdoMetDC [107], an A to C point mutation in the internal ORF initiation codon was not sufficient to completely abolish the increased AdoMetDC expression brought about by spermine depletion. The apparent discrepancy between these results and the results obtained with reporter plasmids may be explained by the stabilization of the AdoMetDC protein in response to spermine depletion. Thus, when the AdoMetDC 5'UTR containing the same point mutation was fused to either luciferase or growth hormone, changes in polyamine concentrations had no effect on plasmid expression [107,113].

In contrast to the reduction in AdoMetDC synthesis seen in T-cells, the regulation in response to changes in intracellular levels of polyamines is not affected by the position of the internal ORF in the 5'UTR, since extending the distance between the cap and the internal ORF to 69 nucleotides had no effect [113]. Thus, changes in polyamine levels do not change the efficiency of recognition of the internal ORF. It is also noteworthy that polyamine regulation is conferred when the internal ORF is inserted into the human growth hormone 5'UTR, suggesting that a protein which does not normally respond to polyamines can be made responsive by the insertion of this ORF sequence within its 5'UTR [113]. This hypothesis has not yet been tested with other proteins.

Quite remarkably, it appears that the amino acid sequence that is encoded by the upstream ORF is critical for both cell-specific regulation of AdoMetDC translation and regulation by polyamines. The 18 nucleotide ORF potentially codes

Table 1
Influence of the sequence of the AdoMetDC internal ORF on translational regulation

Nucleic acid sequence of internal ORF	Amino acid sequence encoded	Polyamine regulation in CHO cells	Polysome size in T-cells
ATG GCC GGC GAC ATT AGC TAG	M A G D I S*	yes	monosome
ATG AGC GGC GAC ATT AGC TAG	M E G D I S*	ND	monosome
ATG GCC GCC GAC ATT AGC TAG	M A A D I S*	ND	monosome
ATG GCC GGC CGC ATT AGC TAG	M A G R I S*	no	medium
ATG GCC GGC GAC GCT AGC TAG	M A G D A S*	no	small
ATG GCC GGC GAC ATT GCT TAG	M A G D I A*	no	medium
ATG GCA GGG GAT ATC TCC TAG	M A G D I S*	yes	monosome

The results shown are a summary of those reported in Refs. [105,113]. Shown are mutations made in the nucleotide sequence of the internal ORF and the amino acid sequence of the peptide product. Transient transfections were performed in Jurkat cells or in CHO cells with or without the addition of 5 mM DFMO. ND = not determined.

for the hexapeptide MAGDIS. Alteration of the nucleotide sequence by incorporating all of the possible changes that do not change the amino acids encoded did not abolish the regulation by polyamines or the reduction in synthesis in T-lymphocytes. In contrast, changes that altered the final three amino acids eliminated all regulation. A summary of the influence of mutations within the internal ORF on both cell-specific regulation and polyamine regulation is shown in Table 1. These studies indicate that in order for the sequence to confer regulation it must code for a peptide, MXXDIS [105,113]. The cell-specific effect involves regulation of recognition of the internal ORF, while polyamines are thought to regulate interaction of the peptide with its target in a manner that has not yet been defined [108,113].

In summary, the results are consistent with a model in which the upstream ORF in the 5'UTR of AdoMetDC mRNA causes ribosome stalling. This decreases the efficiency with which the downstream ORF encoding AdoMetDC protein is translated. The stalling by the upstream ORF is affected by two factors: the content of cellular polyamines and the peptide sequence encoded by the upstream ORF. High polyamine levels cause stalling to occur more effectively. Experiments in which the cellular content of spermidine or spermine are manipulated individually by the use of appropriate inhibitors suggest that either polyamine can bring about this effect but spermine

may be more effective. The manner by which polyamines interact with the nascent peptide is of great potential interest but is currently unknown.

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